Erythrocyte membrane changes of chorea-acanthocytosis are the result of altered Lyn kinase activity

De Franceschi, L ; Tomelleri, C ; Matte, A ; Brunati, A M ; Bovee-Geurts, P H ; Bertoldi, M ; Lansoder, E ; Tibaldi, E ; Danek, A ; Walker, R H ; Jung, H H ; Bader, B ; Siciliano, A ; Ferru, E ; Mohandas, N ; Bosman, G J

Abstract: Acanthocytic red blood cells (RBCs) are a peculiar diagnostic feature of chorea-acanthocytosis (ChAc), a rare autosomal recessive neurodegenerative disorder. Although recent years have witnessed some progress in the molecular characterization of ChAc, the mechanism(s) responsible for generation of acanthocytes in ChAc is largely unknown. As the membrane protein composition of ChAc RBCs is similar to that of normal RBCs, we evaluated the tyrosine-(Tyr)-phosphorylation profile of RBCs using comparative proteomics. Increased Tyr-phosphorylation state of several membrane proteins including band 3, beta-spectrin and adducin was noted in ChAc RBCs. In particular, band 3 was highly phosphorylated on the Tyr-904 residue, a functional target of Lyn, but not on Tyr-8, a functional target of Syk. In ChAc RBCs band 3 Tyr-phosphorylation by Lyn was independent of the canonical Syk mediated pathway. The ChAc-associated alterations in RBC-membrane-protein organization appear to be the result of increased Tyr-phosphorylation leading to altered linkage of band 3 to the junctional complexes involved in anchoring the membrane to the cytoskeleton as supported by co-immunoprecipitation of -adducin with band 3 only in ChAc RBC-membrane treated with the Lyn-inhibitor PP2. We propose this altered association between membrane skeleton and membrane proteins as novel mechanism in the generation of acanthocytes in ChAc.

DOI: https://doi.org/10.1182/blood-2011-05-355339

Erythrocyte membrane changes of chorea-acanthocytosis are the result of altered Lyn kinase activity

Lucia De Franceschi[1], Carlo Tomelleri[1,*], Alessandro Matte[1,*], Anna Maria Brunati[3], Petra H Bovee-Geurts[2], Mariarita Bertoldi[4], Edwin Lasonder[5], Elena Tibaldi[3], Adrian Danek[6], Ruth H Walker[7], Hans H Jung[8], Benedikt Bader[6], Angela Siciliano[1], Emanuela Ferru[1,8], Narla Mohandas[10], Giel JCGM Bosman[2]

[1]Department of Medicine, University of Verona, Verona; Italy;
[2]Department of Biochemistry and Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;
[3]Department of Biochemistry, University of Padova, Padova; Padova, Italy;
[4]Department of Life and Reproduction Sciences, Section of Biochemistry, University of Verona, Verona; Italy
[5]Centre for Molecular and Biomolecular Informatics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[6]Dept of Neurology, Ludwig Maximilians Universität, Munich, Germany;
[7]Department of Neurology, James J Peters VAMC, Bronx, New York, and Mount Sinai School of Medicine, New York, USA;
[8]University Hospital of Zurich, Switzerland
[9]Department of Genetic, Biology and Biochemistry, University of Torino,Torino; Italy
[10]New York Blood Center, New York, NY, USA

[*] These two Authors have equally contributed to the paper

Key words: erythrocyte; membrane; neuroacanthocytosis; cytoskeleton; signaling; phosphorylation.

Running title: Syk independent Lyn activation

Corresponding Author:
Lucia De Franceschi, MD
Dept. of Medicine, University of Verona
P.le L Scuro, 10, 37134 Verona; Italy
E-mail: lucia.defranceschi@univr.it; FAX +390458027473; phone: +390458124918

ABSTRACT

Acanthocytic red blood cells (RBCs) are a peculiar diagnostic feature of chorea-acanthocytosis (ChAc), a rare autosomal recessive neurodegenerative disorder. Although recent years have witnessed some progress in the molecular characterization of ChAc, the mechanism(s) responsible for generation of acanthocytes in ChAc is largely unknown. As the membrane protein composition of ChAc RBCs is similar to that of normal RBCs, we evaluated the tyrosine-(Tyr)-phosphorylation profile of RBCs using comparative proteomics. Increased Tyr-phosphorylation state of several membrane proteins including band 3, beta-spectrin and adducin was noted in ChAc RBCs. In particular, band 3 was highly phosphorylated on the Tyr-904 residue, a functional target of Lyn, but not on Tyr-8, a functional target of Syk. In ChAc RBCs band 3 Tyr-phosphorylation by Lyn was independent of the canonical Syk mediated pathway. The ChAc-associated alterations in RBC-membrane-protein organization appear to be the result of increased Tyr-phosphorylation leading to altered linkage of band 3 to the junctional complexes involved in anchoring the membrane to the cytoskeleton as supported by co-immunoprecipitation of β-adducin with band 3 only in ChAc RBC-membrane treated with the Lyn-inhibitor PP2. We propose this altered association between membrane skeleton and membrane proteins as novel mechanism in the generation of acanthocytes in ChAc.

INTRODUCTION

Chorea-acanthocytosis (ChAc) is a rare autosomal recessive neurodegenerative disorder of the neuroacanthocytosis (NA) group. [1-3] ChAc is characterized by a neurodegeneration syndrome of the basal ganglia that is associated with the presence of acanthocytes, abnormal erythrocytes with thorn-like protrusions, in the peripheral circulation. [1-3] Molecular studies have identified mutation(s) of the *VPS13A* gene (chromosome 9), encoding a 360 kDa protein chorein of unknown function that is ubiquitously expressed in the brain. [2,4-6] To date 92 mutations on the *VPS13A* gene have been reported, resulting in low or absent synthesis of chorein or in expression of a functionally defective protein at normal levels. [2,4-6] Chorein has been detected in mature red cells but it is partially or completely absent in red cells from patients with ChAc. [6] Due to the lack of knowledge on the structure of chorein and on its interaction with other proteins, we can only speculate on the role of chorein in red cell homeostasis.

In the red cell membrane two major multiprotein complexes bridge the lipid bilayer with the integral membrane proteins to the spectrin-actin cytoskeleton: the ankyrin complex and the junctional or 4.1R complex.[7] Electron microscopy of ChAc red cells reveals ultrastructural abnormalities in the membrane skeleton as indicated by a heterogeneous distribution of the cytoskeleton. Condensed skeletal structures around protrusions and a less filamentous structure in some large membrane patches indicate a perturbation of membrane cytoskeleton network associated with the membrane protrusions that characterize acanthocytes.[8] These structural data were also supported by the observation that ChAc patients have a fraction of dense red cells containing acanthocytes with a reduced cell K^+ content compared to normal controls.[9] Red cells from ChAc patients did not show overall abnormalities in red cell membrane protein composition and content; although there is accumulating evidence for neuroacanthocytosis-specific abnormalities in band 3 structure and function.[10] Increased membrane serine-threonine phosphorylation levels mainly of band 3 and spectrin, increased levels of band 3 tyrosine phosphorylation and increased activity of membrane associated casein-kinase have also been described in ChAc red cells.[11] Increased amount of N^{ε} (γ-glutamyl) lysine isopeptides associated with the red cell membrane has also been reported in a few ChAc patients, indicating a possible perturbation of the anchoring bridges between the membrane and the cytoskeleton.[12,13] There is no consistent data indicating a relation between lipid content and/or composition and acanthocytosis in ChAc patients.

The recent development of proteomic techniques has yielded a description of red cell membrane proteome mainly in normal red cells.[14-18] We used comparative proteomics to study membrane proteome of red cells from patients with ChAc in relation to normal controls. Differences between normal and ChAc red cells included changes in tyrosine phosphorylation state of band 3, beta spectrin and other members of the anchoring complexes in ChAc red cells. We also found abnormal activation of Lyn, a Tyr-kinase of the Src family, independent from its canonical signaling pathway involving primary phosphorylation of Syk. These finding suggest that altered phosphorylation of band 3 and membrane skeletal proteins may play a role in the development of acanthocyte morphology in chorea-acanthocytosis.

MATERIALS AND METHODS

Design of the study.

We studied nine patients with chorea-acanthocytosis (ChAc) based on clinical neurological manifestations, presence of acanthocytes (Fig. 1a) and confirmation by either immunoblot analysis for chorein and/or molecular analysis for *VPS13A* mutations as previously described.[6] The demographic and molecular data of patients are reported in Table 1. Blood was obtained by venipuncture using EDTA as an anti-coagulant, according to the guidelines approved by the local Ethics Committees for human subject studies of all participating institutions involved. Blood samples (ChAc patients and healthy control donors) were shipped to Verona and Nijmegen at 4°C, and processed immediately after arrival. Erythrocytes were isolated from whole blood after removal of white blood cells and platelets as previously described.[19] Since ChAc patients have a dense fraction of red cells enriched in acanthocytes,[9] we analyzed red cells separately according to red cell density.

For semi-quantitative proteome analyses, we separated the red cells in one of two ways. Cells were separated into five fractions (I-V) based on volume and density using counter flow centrifugation followed by Percoll gradient centrifugation, with fraction I containing the youngest, and fraction V the oldest erythrocytes and acanthocytes. The membrane proteomes of these fractions were analyzed as described previously.[15]

Alternatively, red cells were separated into two fractions: fraction 1 (F1) corresponding to a density < 1.074, containing reticulocytes, and fraction 2 (F2) corresponding to a density > 1.092, containing acanthocytes, for proteomic analysis by 2DE-MALDI-TOF MS, which requires a relatively large amount of proteins for the analysis.[19,20] Where indicated control red cells were incubated in the presence of sodium orthovanadate as previously described by Bordin et al.[21]

Red cell morphology, membrane preparation and membrane cytoskeleton extraction

To study cell morphology, red cells were fixed as previously described by Matte et al.[22] and imaged using a Leica TCS SP5 II microscope equipped with an Objective HCX PL APO lambda blue 63.0x, NA 1.40 oil.

Red cell membrane fractions were obtained by lysing one volume of packed cells in 10 volumes of ice-cold phosphate lysis buffer (PLB, 5 mM Na_2HPO_4 pH 8.0, in the presence of protease inhibitors (cocktail tablet, Roche; Germany), 3 mM benzamidine, 1 mM

Na_3VO_4). Samples were incubated for 10 min on ice and centrifuged for 10 min at 12,000 x g at 4°C. Membranes were then washed four times by centrifugation at 12,000 x g at 4°C with PLB until they appeared almost white.[15]

To assess the strength of the interaction between cytoskeleton and the lipid bilayer, cytoskeletal proteins were extracted by resuspending membranes (100 µg of total protein) in one ml of 100 mM Na_2CO_3 pH 11. The suspension was passed five times through a 25-gauge needle, mixed and incubated for 30 min at 4°C. Soluble proteins were removed by centrifugation for 30 min at 200,000 x g. This procedure was repeated twice. Proteins from pelletted membranes were extracted by either detergent or NaCl. Briefly, packed erythrocyte membranes were incubated for 1 hour at 4°C with 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM sodium orthovanadate, and protease inhibitor cocktail in the absence or the presence of either 1% Triton X-100 [22] or 0.6 M NaCl. After ultracentrifugation, the supernatants and precipitates were analysed by one-dimensional electrophoresis and either stained with colloidal Coomassie or transferred to membranes for immunoblot analysis with the appropriate antibody.[22]

Comparative proteomic analysis

Semi-quantitative proteomic analysis. All the analyses were performed essentially as previously described.[15] Details are reported in Supplementary Methods section. Samples of various origins were quantified by the label-free peptide counting method emPAI, that calculates protein abundancy, as has been applied for a quantitative analysis of RBC membranes during storage.[15] Data were normalized using the median emPAI value per sample. This protocol has been shown to result in a reliable identification of all erythrocyte membrane proteins, and to enable semi-quantification of the major protein species.[15] In order to be able to apply this method to multiple samples in an efficient and cost-effective manner, we reduced the running time of the SDS-PAGE step and extended the running time of the HPLC gradient from 1.5 to 4 hours. This 'one-slice' method yielded 300-400 reliably identified proteins, comparable to those found earlier by us and others using one-dimensional as well as two-dimensional electrophoresis.[15,23,24]

Gel electrophoresis of red cells membrane. For one-dimensional electrophoresis (1DE), red cell membranes were solubilized in sample buffer (50 mM Tris pH 6.8, 100 mM β-mercaptoethanol, 2% v/v SDS, 10% v/v glycerol, a few grains of bromophenol blue), and loaded on to either 8 % or 10 % polyacrylamide gels. Whenever peroxiredoxin-2 (Prx-2) was evaluated, red cells were lysed in the presence of NEM (100 mM) in the PLB and solubilized in sample buffer with NEM (100 mM).[19,20,22] For two-dimensional

electrophoresis (2DE), red cell membranes were delipidated and separated by 2DE as previously described. [19,20,22] Details are presented in Supplementary Methods section After electrophoresis the gels were stained with colloidal Coomassie followed by image analysis using "Progenesis SameSpots" software (Non Linear Dynamics, Newcastle-Upon-Tyne, UK).[20] Details on image analysis are reported in Supplementary Methods section. The selected bands or spots were identified by MALDI-TOF MS/MS analysis (see in Supplementary Methods section for more details).

Immunoblot analysis and dephosphorylation of blotted proteins

Proteins were transferred from 1D or 2D gels to membranes for immunoblot analysis and probed with specific antibodies as previously described,[19,20] see also Supplementary Methods for details. Dephosphorylation of blotted proteins was carried out as described in Supplementary Methods section.

Peptides and recombinant proteins

The phosphopeptide of band 3 (aa 1-26) and its unphosphorylated analogue were synthesized as described previously.[25] The recombinant GST-Lyn/SH3 domain was expressed and purified according to the protocol described by Trentin et al. [26] The GST-Lyn/SH2 domain was expressed and purified as previously reported. [27]

Phosphorylation of erythrocyte membranes

Erythrocyte membranes (3 µg protein) were phosphorylated for 10 minutes at 30°C in 30 µL of an incubation mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 30 µM ATP, 200 µM sodium orthovanadate and 15 nM of exogenous Lyn tyrosine kinase, purified from rat spleen.[27] When required, the phosphorylation assays were carried out in the absence or presence of the GST-Lyn/SH2 domain or the GST-Lyn/SH3 domain, respectively. The solubilized membranes were analyzed by immuno-blot with the anti- p-Tyr antibody. The membranes were reprobed with anti-β-actin antibody as a loading control. When indicated washed red cells from control and ChAc subjects were incubated with and without the Src family kinase inhibitors PP1 (10 µM) and PP2 (10 µM) as previously reported.[19,27]

Preparation of Syk-phosphorylated membranes of erythrocytes

Membranes were phosphorylated by 15-nmol/L $p36^{Syk}$ for 10 minutes in 50 mM Tris-HCl, 10 mM $MnCl_2$, 30µM ATP and 200 µM sodium orthovanadate. Syk-phosphorylated membranes were separated from $p36^{Syk}$, ATP, and other reagents by centrifugation and washed twice as previously reported.[27]

Identification of putative phosphorylation targets of Lyn by sequence and structure analyses

The sequences of the different Tyr-phosphorylated proteins were obtained from the NCBI reference sequence database using their accession numbers. They were analyzed by means of the GPS 2.0 software to identify putative Lyn target sites.[28] Details are reported in Supplementary Methods section.

RESULTS

Proteomic analysis of ChAc red cell membranes shows differences compared with membranes from healthy controls

Analysis of ChAc red cell membrane proteins by one-dimensional electrophoresis followed by protein staining showed that the membrane protein composition of ChAc red cells was very similar to that of red cells from healthy controls except for a band at ~ 20 kDa, which was markedly reduced in the high density (F2) fraction of red cells of ChAc patients (Fig. 1b). This band was excised from the gel and identified by mass spectrometry as peroxiredoxin-2 (Prx2). Analysis of the red cell membrane protein composition by comparative semi-quantitative proteomic approach showed an enrichment in ChAc red cell membranes for spectrin and for ankyrin and protein 4.2 major component of the ankyrin complex (Supplementary Table 1S). The concentrations of the raft-associated flotillins did not differ between control and ChAc samples. However, stomatin, another raft-associated protein, was conspicuously enriched in the ChAc samples (Supplementary Table 1S). The membrane association of GAPDH was variable with an increase in two out of three ChAc patients and a decrease in one ChAc patient (Supplementary Table 1S). In addition, proteins of the small G protein family were hardly detectable in the control samples but were abundant in the patients' membrane fractions. All membrane samples contained similar amounts of heat shock proteins (data not shown). Immunoblot analysis with specific antibodies confirmed: (I) a reduction of membrane associated Prx2 in both low and high density fractions of ChAc red cells compared to controls; (II) an increased membrane association of GAPDH in the high density fraction of ChAc red cells that were enriched in acanthocytes compared to normal controls; (III) a similar membrane content of flotillin 1 in ChAc and control red cells (Fig. 1c).

For a more detailed analysis, we performed two-dimensional electrophoresis (2DE) followed by identification of the differently expressed proteins by mass spectrometry. Using a twofold abundance as the selection criterion (see also Materials and Methods) we detected 50 spots that were differently expressed in high density fraction (F2) of ChAc red cells and 41 spots differently expressed in low density fraction (F1) of ChAc red cells (Fig. 1d, Supplementary Table 2S). It is to be noted that our protein identifications are in strong accordance with previously published 2DE maps of red cell membrane.[14,20] The identified

proteins were divided into eight functional-clusters; (I) proteins of the anchoring complexes; (II) cytoskeleton proteins; (III) raft-related proteins; (IV) membrane channels and transporters; (V) intracellular signaling proteins; (VI) metabolic enzymes; (VII) proteins of the ubiquitin-proteasome system and (VIII) stress response proteins and chaperones.[20] We validated the 2DE data by immunoblot analysis of some of the identified proteins: protein p55 in F1 fractionated red cells, flotillin-1 in F1 fractionated red cells and protein 4.1 in F2 fractionated red cells (Fig. 1e). The differences in membrane and cytoskeleton proteins in ChAc red cell observed in 2DE maps were mainly related to protein 2DE mobility shift, possibly reflecting protein post-translational modifications (PTMs), such as phosphorylation (Fig. 1d-e and Supplementary Table 2S). This is also supported by the similar abundancy of membrane and cytoskeleton proteins in 1DE analysis of ChAc and control red cell membranes (Fig. 1b).

Differential extraction of membrane proteins reveals differences in membrane organization in ChAc red cells

In order to explore putative ChAc-associated changes in membrane organization, we assessed the strength of binding of the major erythrocyte membrane and cytoskeletal proteins to the lipid bilayer by analyzing the proteome following incubation of the membrane fractions with 100 mM Na_2CO_3, pH 11 (see Materials and Methods). In controls, this alkaline treatment led to the virtual disappearance of most cytoskeletal and membrane-associated proteins such as spectrin, ankyrin, and GAPDH. This was accompanied by enrichment of most integral membrane proteins including band 3 and Glut1, and by a large increase in the concentration of stomatin (Table 3S). The loss of GPC but not GPA suggests that a considerable fraction of the GPC molecules is extracted together with the cytoskeleton. As in control samples, extraction of ChAc membranes resulted in the depletion of the same proteins as seen in normal membranes and also in enrichment for most integral membrane proteins with band 3 as a notable exception (Table 3S).

Since recent studies have suggested that changes in membrane protein phosphorylation state plays an important role in the modulation of red cell membrane protein-protein functional cross-talk and stability of multi-protein structures, [29,30] we studied the tyrosine (Tyr-) phosphorylation profile of red cell membrane from control and ChAc subjects.

The tyrosine-phosphorylation state of ChAc red cell membrane proteins is increased compared to normal controls

Evaluation of the Tyr-phosphorylation pattern of red cell membranes from normal and

ChAc density-separated red cells by immunoblot analysis showed higher Tyr-phosphorylation state of various membrane proteins in ChAc red cells (Fig. 2a-b).
The 2DE Tyr-phospho maps confirmed the presence of trains of Tyr-phosphorylated proteins in the gel area corresponding to the differently expressed spots noted in 2DE maps: ie.: ankyrin (17-18C in F1 and 54C-57C and 60C-62C in F2 from ChAc red cells), protein 4.1R (21C, 23C, 25 C in F1 and 64C-68C in F2 from ChAc red cells), protein p55 (32C-34C in F1 from ChC red cells) and dematin (88C-89C in F2 from ChAc red cells) (Fig. 1d and Supplementary Table 2S). These data are also supported by the absence of reactivity when the membranes were treated with λ-phosphatase, which was used to remove phosphate groups from blotted proteins (Fig. 2a, lower panel). The differently Tyr-phosphorylated proteins were excised from the 1D stained gels and identified by mass spectrometry (Fig. 2b). According to their functions the identified proteins were divided into eight functional-clusters: (I) membrane protein, band 3; (II) cytoskeleton network proteins, β-spectrin and β-actin; (III) ankyrin complex proteins, ankyrin, band 4.2; (IV) membrane junctional complex proteins, band 4.1, p55, β-adducin; (V) intracellular signaling protein; (VI) metabolic enzymes such as GAPDH; (VII) stress response proteins such as catalase and (VIII) cell trafficking protein such as Ras-related proteins (Table 2). Of note we found a Tyr-phosphorylated β-spectrin fragment in both F1 and F2 fractions from ChAc patients and we identified a phosphopeptide of β-adducin (R.MLDNLGYR.T) in the dense red cell fraction from ChAc subjects (Table 2). These results suggest that altered phosphorylation may contribute to acanthocytosis of ChAc.

Lyn is abnormally activated in ChAc red cells and is independent from Syk sequential phosphorylation

Since Tyr-kinase Syk and the Src family kinase Lyn have been described to be involved in modulating band 3 function in healthy red cells,[27] we studied membrane association of Syk and Lyn in control and ChAc erythrocytes.
In both F1 and F2 red cells from ChAc patients we observed an increased membrane association of active Lyn (phospho-Lyn, Fig. 3a).In contrast, the amount of membrane-bound total and active Syk (phospho-Syk) was almost undetectable in ChAc and barely detectable in control red cells (Fig. 3 a). We previously reported a sequential phosphorylation of band 3 catalyzed by the concerted action of Syk and Lyn, with Tyr-8 and Tyr-21 being first targeted by Syk, and thereby serving as docking sites for the Src homology domain 2 (SH2) of Lyn. Lyn, once recruited, phosphorylates Tyr-359 and Tyr-

904 of band 3[27] as well as other proteins such as spectrins and adducin (unpublished data).

We used specific antibodies against Tyr-8 on the N-terminal of band 3, as a Syk target, and Tyr-904 on the trans-membrane domain of band 3, as a Lyn target. We found increased Tyr-phosphorylation at Tyr-904 compared to normal red cells (Fig. 3b). Phosphorylation of residueTyr-8 was almost absent in both fractions from ChAc red cells and only slightly detectable in normal red cells. Since we have previously reported that activated Syk might be present in the cytoplasm in a truncated form,[21] we evaluated Syk activity in the cytoplasm. Syk activity was similar in the cytoplasm of normal and ChAc red cells (data not shown), suggesting a peculiar membrane recruitment of Lyn to ChAc membranes that occurs independent of preceding phosphorylation of band 3 by Syk.

To evaluate whether the Tyr-phospho profile of ChAc red cell membrane might be affected by in vitro Lyn inhibition, we incubated control and ChAc red cells with both PP1 and PP2, Src family kinase inhibitors to obtain a more stable inhibition of kinase activity. As shown in Fig. 3c, in ChAc red cells the PP1/PP2 treatment markedly reduced Tyr-phosphorylation state of several bands, including band 3, thus supporting the role of Src kinase as determinant of the peculiar ChAc red cell membrane Tyr-phosphorylation profile.

Since the amount of Lyn was markedly higher in ChAc erythrocytes than in control red cells, we characterized Lyn red cell membrane association. Lyn was almost entirely solubilized by the treatment with Triton-X 100 in both control and ChAc red cells (Fig. 4a, lanes 2-3 and 7-8). However, treatment with high ionic strength medium (0.6 M NaCl, see Materials and Methods) resulted in a remarkably more extraction of Lyn from ChAc red cell membranes compared to that from membranes of control cells, accounting for the increased Lyn concentration (Fig. 4a, lanes 4-5 vs 9-10). These data suggest there are several pools of Lyn in ChAc red cells, which associate with the membrane by multiple modes. This is corroborated by the observation that a fraction of Lyn was released from ChAc red cell membranes by agents capable of disrupting the SH2 domain/phospho-Tyr interaction, such as GST-Lyn/SH2 and the phosphorylated NH2 terminus of band 3 (Fig. 4b).

Exogenous Lyn phosphorylates several targets in ChAC red cell membrane independent of Syk

We then evaluated whether in ChAc red cells the ability of Lyn to phosphorylate red cell membrane proteins *in vitro* was still dependent on a Syk-related mechanism (see above). Preparations from control and ChAc red cell membranes were washed free of the

phosphatase inhibitor vanadate and resealed with exogenous Lyn in the presence and absence of GST-Lyn/SH2 and GST-Lyn/SH3 domains. Exogenous Lyn was shown to phosphorylate ChAc membrane proteins much more efficiently than control fractions (Fig. 5). In addition, the Tyr-phosphorylation profile remained unaltered in the presence of the GST-Lyn/SH3 domain, with only a slight decrease when the GST-Lyn/SH2 domain was added (Fig. 5). These data indicate that the ability of Lyn to Tyr-phosphorylate ChAc membrane proteins is not dependent on a preceding membrane binding and activity of Syk as previously reported for control red cells.[27] Thus, ChAc-associated alterations in the membrane protein organization may allow Lyn to target its substrates independently from Syk. Interestingly, exogenous Lyn promotes Tyr-phosphorylation of membrane proteins other than band 3, similarly to that observed in the naturally occurring Tyr-phosphorylation profile of ChAc red cell membranes (Fig. 3a and Fig. 5). It is of note that in vitro phosphorylation of ChAc red cell membrane by exogenous Lyn involved a smaller number of proteins (bands) at molecular weight lower than 48 kDa compared to intact ChAc red cells. Otherwise, these proteins showed reduced Tyr-phosphorylation in PP1/PP2 treated ChAc red cells, suggesting that Lyn might be also part of other signalling pathways involving other kinases or play a role as downstream regulator of phosphatase(s) similarly to what we previously described in other red cell models.[31,32]

Bioinformatic analysis of the differentially Tyr-phosphorylated proteins reveals Lyn targets in ChAc red cells

To evaluate the presence of possible Lyn targets on ChAc red cell membrane proteins differentially Tyr-phosphorylated, we carried out an extensive bioinformatic analysis of putative Lyn target sites in the Tyr-phosphorylated proteins identified. This analysis reveals that approx. 75% of the proteins has at least one highly Lyn-selective site (Table 3). Only 3 of them (aldehyde dehydrogenase, carbonic anhydrase and glyceraldehyde dehydrogenase) show low specificity (bold) and 2 (stomatin and rab) an even lower specificity for Lyn (italic), with increased specificity for other Src kinase members (data not shown). Since we found a highly phosphorylated β-spectrin peptide in the proteomic analysis and also identified a β-adducin phosphopeptide, we carried out a sequence structure analysis of β-spectrin or β-adducin to further characterize the Lyn targets in these two proteins. The analysis of the sequence of β-spectrin for Lyn-directed peptides with a high threshold score > 2.4 identified five peptides (Table 3, light grey). An intercrossed search to identify if these peptides could also serve as substrates for Src tyrosine kinases restricted the Lyn-specific target sequences to two tyrosine residues: Tyr-307 and Tyr-

1590. Inspection of the 3D-structure, indicated that the Tyr-307 is located inside the triplie helical bundle, while Tyr-1590 is in the linker region preceding repeat 14, at the interface of the ankyrin binding domain (repeats 14-15) [33-35] (Table 3, light grey). The phosphopeptide of β-adducin (Tyr-377) is a specific target of Lyn with little specificity for Src (Table 3, light grey) and is located in a highly conserved sequence of the neck domain of the protein.[36]

Lyn-mediated phosphorylation of ChAc membranes alters the interaction of band 3 with β-adducin

We then evaluated the role of Lyn-dependent phosphorylation of band 3 in the interaction with β-adducin in ChAc red cells compared to normal erythrocytes. We first phosphorylated band 3 by exogenous Syk with or without exogenous Lyn using normal red cell membranes, then extracted by Triton-X (Fig. 6a). The immunoprecipitated band 3 from the resulting extract was further subjected to immunoblot analysis with anti-adducin antibody. As shown in Fig. 6b, in control erythrocytes the presence of β-adducin in the band 3 immunoprecipitate was dramatically decreased only if the membranes were sequentially phosphorylated by Syk and Lyn. Since in ChAc red cells we have shown band 3 Tyr-phosphorylated by Lyn independently from Syk phosphorylation, we immunoprecipitated band 3 from ChAc extracts and we observed small amount of β-adducin co-immunoprecipitated, which increased further when membranes were incubated in the presence of the Src kinase inhibitor PP2, suggesting that the altered Lyn activity was implicated in the mechanism of association between band 3 and β-adducin in ChAc red cells.

DISCUSSION

An integrated proteomic approach enabled us to analyze the perturbation of membrane protein organization in ChAc red cells at the molecular level. Our analysis indicated that this membrane perturbation mainly affects the multiprotein complexes that anchor the cytoskeleton to the membrane through band 3. The ChAc-related alterations in the membrane association of GAPDH and hemoglobin also suggest a perturbation of their binding sites on band 3. These observations are in agreement with the reduction in association of Prx2 with the membrane, that may be due to unavailability of the Prx2 docking site on band 3, as has also been reported in β-thalassemic red cells.[22] These data confirm and extend previous observations of neuroacanthocytosis-associated alterations on band 3[10] and extend these to chorea-acanthocytosis specific alterations in band 3 function.

Previous studies have shown that Tyr-phosphorylation state of red cell membrane proteins is involved in regulation of membrane cohesion and mechanical stability.[29] Accordingly, we found that several membrane proteins are highly Tyr-phosphorylated in ChAc red cells as compared to controls. These proteins are band 3 and other components of the two anchoring complexes. Since band 3 is part of both complexes, these findings suggest that phosphorylation-induced perturbation of protein-protein interactions may be centered on band 3. Changes in the protein phosphorylation state can modify protein-protein interactions and thereby multiprotein complex formation and/or functions.[37,38]

Previous studies which documented that the Syk-Lyn signaling pathway working in concert can modify band 3 function in healthy red cells.[27] It is noteworthy that we found increased association of active Lyn with the ChAc membranes in the absence of Syk. This is surprising, since Syk is responsible for the primary tyrosine phosphorylation of band 3 that precedes membrane recruitment of Lyn in normal red cells.[27] In ChAc membranes the membrane-associated form of Lyn was normally solubilized by detergent but differentially partitioned by ionic strength extraction. These data point towards a contribution of electrostatic interactions in Lyn binding and suggest the existence of different pools of Lyn associated with the membrane. A small amount of Lyn was displaced from the ChAc membrane by the addition of GST-Lyn/SH2 or the phosphorylated cytoplasmic domain of band 3. This suggests the unmasking of the Lyn domain on band 3, which allows Lyn to bind independently from the action of Syk. The conspicuous increase in Tyr-

phosphorylation state of ChAc red cell membrane proteins by exogenous Lyn supports this hypothesis.

Bioinformatic analyses show that the large part the differentially Tyr-phosphorylated proteins identified in ChAc indeed contain Lyn-specific targets or putative Src kinase family substrates. In addition, the sequence-structure study allowed us to propose specific Lyn phosphorylation sites on β-adducin and β-spectrin, supporting the multi-target action of abnormally activated Lyn in ChAc red cells. Notably, Tyr-377 of β-adducin, that is phosphorylated in ChAc red cells, is highly conserved.[36] This residue is located in the neck region that is involved in association of adducin monomers to heterodimers, thereby constituting the functionally active form of the protein. This conserved sequence in the neck region of adducin is also involved in the organization of an amphiphilic structure, which is important in mediating protein-protein interactions.[39] In ChAc red cells β-adducin was coimmunoprecipitated with band 3 only in presence of the Src kinase inhibitor PP2, while in control erythrocyte there was a requirement for sequentially Syk-Lyn band 3 phosphorylation. Thus, changes in the Tyr-phosphorylation state of β-adducin in this region are likely to affect its interactions with the other proteins of the junctional complexes and thereby the stability of the red cell membrane protein bridges.

Our data suggest a novel mechanism in generation of acanthocytes in ChAc in addition to what was reported in McLeod syndrome, a neuroacanthocytosis related disorder, and in abetalipoproteinemia. In these diseases the acanthocytes formation has been related to either the absence of the XK membrane protein convalently linked to the Kell glycoprotein and part of the multiprotein 4.1 juctional complex as in McLeod syndrome or to the abnormal membrane lipid composition (increased sphingomiein/lecithin ratio) with abnormalities of lipid-lipid interaction within membrane as in abetaliproteinemia.[40,41] In ChAc red cells there is no indication of abnormalities in either membrane lipid composition or membrane-and-cytoskeletal protein content. We did, however, find alterations in Tyr-phosphorylation state of membrane proteins and in signal transduction pathway. Thus, we propose that in ChAc red cells the "Lyn storm" might affect the organization of the complexes bridging the membrane to the cytoskeleton, most likely by inducing abnormal protein interactions through the opening of SH2 binding sites in a Syk-independent manner. This might result in the heterogeneous distribution of the cytoskeleton as observed in electron microscopy studies,[8] and may be similar to the concentration of Lyn in plasma membrane patches of activated platelets, adjacent to granules.[42,43] In addition, abnormal Lyn phosphorylation might also contribute to altered vesiculation of ChAc red

cells. Lyn has been found in association with the exosomes that are excreted by reticulocytes, containing lipid raft domains, and in vesicles from mature red cells.[44-46] Together with the indication for a stomatin-specific, raft-based process in vesicle formation in vitro,[47] this supports the hypothesis that vesicle formation is an important, active process in the formation of acanthocytes. This is in agreement with increased concentrations in ChAc red cell membranes of various small-G-proteins involved in vesicle formation and transport.[10]

In conclusion, our data link, for the first time, the presence of abnormal red cells in patients with ChAC with evidence for an independent and strong Lyn activation. The ChAc-associated alterations in red cell membrane-protein organization appear to be the result of increased Tyr-phosphorylation leading to altered linkage of band 3 to the junctional complexes involved in anchoring the membrane to the cytoskeleton. The latter may promote an increased degree of freedom of the junctional complexes bridging the membrane to the cytoskeleton and affect the association of the cytoskeleton with the plasma membrane. The present data open new ways to explore the pathobiology of acanthocytes and pathophysiological mechanisms of neurodegeneration in patients with neuroacanthocytosis.

ACKNOWLEDGEMENTS

The work of was supported by the Advocacy for Neuroacanthocytosis Patients (BB, PB, EL, GB, LDF), Carl H. and Elizabeth S. Pforzheimer III, New York (BB, PB, EL,GB, RHW), Ginger and Glenn Irvine London (PB, EL, GB) and Telethon grant GPP07007 to LDF. Preliminary data were generated by Dr. Christan Pozzobon.

AUTHORS CONTRIBUTION

LDF, GB designed the experiments and analyzed the data; NM contributed to study design and the writing of the manuscript; CT, LDF, GB, PB-G, AM performed the experiments; MB performed the protein structure analysis and contributed to writing of the manuscript; AMB, TE: performed part of the Lyn analysis, analyzed the data and contributed in writing the manuscript; AD, RW, BB performed neurological diagnosis; AD and BB provided blood samples, confirmed diagnosis; EF performed part of the analysis by mass spectrometry EL, CT performed bioinformatic analysis of the proteomic data; all authors reviewed the manuscript. The authors have no financial conflicts of interest to disclose.

REFERENCES

1. Danek A, Jung HH, Melone MA, Rampoldi L, Broccoli V, Walker RH. Neuroacanthocytosis: new developments in a neglected group of dementing disorders. *J Neurol Sci*. 2005;229-230:171-186.
2. Danek A, Walker RH. Neuroacanthocytosis. *Curr Opin Neurol*. 2005;18(4):386-392.
3. Jung HH, Danek A, Frey BM. McLeod syndrome: a neurohaematological disorder. *Vox Sang*. 2007;93(2):112-121.
4. Rampoldi L, Danek A, Monaco AP. Clinical features and molecular bases of neuroacanthocytosis. *J Mol Med*. 2002;80(8):475-491.
5. Velayos-Baeza A, levecque, C., Dobson-Stone, C., Monaco, A.P. The function of chorein. In: Walker RH, Saiki, S., Danek, A., ed. Neuro-acanthocytosis syndromes II. Berlin Heildelberg: Springer-Verlag; 2008:87-105.
6. Dobson-Stone C, Velayos-Baeza A, Filippone LA, et al. Chorein detection for the diagnosis of chorea-acanthocytosis. *Ann Neurol*. 2004;56(2):299-302.
7. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. *Blood*. 2008;112(10):3939-3948.
8. Terada N, Fujii Y, Ueda H, et al. Ultrastructural changes of erythrocyte membrane skeletons in chorea-acanthocytosis and McLeod syndrome revealed by the quick-freezing and deep-etching method. *Acta Haematol*. 1999;101(1):25-31.
9. Clark MR, Aminoff MJ, Chiu DT, Kuypers FA, Friend DS. Red cell deformability and lipid composition in two forms of acanthocytosis: enrichment of acanthocytic populations by density gradient centrifugation. *J Lab Clin Med*. 1989;113(4):469-481.
10. Bosman GJCGM, de Franceschi L. Neuroacanthocytosis-related changes in erythrocyte membrane organization and function. In: Walker RH SS, Danek A, ed. Neuroacanthocytosis syndromes II. Berlin, Germany: Springer; 2008.
11. Olivieri O, De Franceschi L, Bordin L, et al. Increased membrane protein phosphorylation and anion transport activity in chorea-acanthocytosis. *Haematologica*. 1997;82(6):648-653.
12. Murthy SN, Wilson J, Zhang Y, Lorand L. Residue Gln-30 of human erythrocyte anion transporter is a prime site for reaction with intrinsic transglutaminase. *J Biol Chem*. 1994;269(36):22907-22911.
13. Melone MA, Di Fede G, Peluso G, et al. Abnormal accumulation of tTGase products in muscle and erythrocytes of chorea-acanthocytosis patients. *J Neuropathol Exp Neurol*. 2002;61(10):841-848.
14. Pasini EM, Kirkegaard M, Mortensen P, Lutz HU, Thomas AW, Mann M. In-depth analysis of the membrane and cytosolic proteome of red blood cells. *Blood*. 2006;108(3):791-801.
15. Bosman GJ, Lasonder E, Luten M, et al. The proteome of red cell membranes and vesicles during storage in blood bank conditions. *Transfusion*. 2008;48(5):827-835.
16. Bosman GJ, Willekens FL, Werre JM. Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem*. 2005;16(1-3):1-8.
17. Pasini EM, Lutz HU, Mann M, Thomas AW. Red blood cell (RBC) membrane proteomics--Part II: Comparative proteomics and RBC patho-physiology. *J Proteomics*. 2010;73(3):421-435.
18. Pasini EM, Lutz HU, Mann M, Thomas AW. Red blood cell (RBC) membrane proteomics--Part I: Proteomics and RBC physiology. *J Proteomics*. 2010;73(3):403-420.
19. De Franceschi L, Biondani A, Carta F, et al. PTPepsilon has a critical role in signaling transduction pathways and phosphoprotein network topology in red cells. *Proteomics*. 2008;8(22):4695-4708.

20. Siciliano A, Turrini F, Bertoldi M, et al. Deoxygenation affects tyrosine phosphoproteome of red cell membrane from patients with sickle cell disease. *Blood Cells Mol Dis*. 2010;44(4):233-242.
21. Bordin L, Ion-Popa F, Brunati AM, Clari G, Low PS. Effector-induced Syk-mediated phosphorylation in human erythrocytes. *Biochim Biophys Acta*. 2005;1745(1):20-28.
22. Matte A, Low PS, Turrini F, et al. Peroxiredoxin-2 expression is increased in beta-thalassemic mouse red cells but is displaced from the membrane as a marker of oxidative stress. *Free Radic Biol Med*. 2010;49(3):457-466.
23. Bosman GJ, Lasonder E, Groenen-Dopp YA, Willekens FL, Werre JM, Novotny VM. Comparative proteomics of erythrocyte aging in vivo and in vitro. *J Proteomics*. 2010;73(3):396-402.
24. van Gestel RA, van Solinge WW, van der Toorn HW, et al. Quantitative erythrocyte membrane proteome analysis with Blue-native/SDS PAGE. *J Proteomics*. 2010;73(3):456-465.
25. Donella-Deana A, James P, Staudenmann W, et al. Isolation from spleen of a 57-kDa protein substrate of the tyrosine kinase Lyn. Identification as a protein related to protein disulfide-isomerase and localisation of the phosphorylation sites. *Eur J Biochem*. 1996;235(1-2):18-25.
26. Trentin L, Frasson M, Donella-Deana A, et al. Geldanamycin-induced Lyn dissociation from aberrant Hsp90-stabilized cytosolic complex is an early event in apoptotic mechanisms in B-chronic lymphocytic leukemia. *Blood*. 2008;112(12):4665-4674.
27. Brunati AM, Bordin L, Clari G, et al. Sequential phosphorylation of protein band 3 by Syk and Lyn tyrosine kinases in intact human erythrocytes: identification of primary and secondary phosphorylation sites. *Blood*. 2000;96(4):1550-1557.
28. Xue Y, Ren J, Gao X, Jin C, Wen L, Yao X. GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol Cell Proteomics*. 2008;7(9):1598-1608.
29. Pantaleo A, De Franceschi L, Ferru E, Vono R, Turrini F. Current knowledge about the functional roles of phosphorylative changes of membrane proteins in normal and diseased red cells. *J Proteomics*. 2010;73(3):445-455.
30. Ferru E, Giger K, Pantaleo A, et al. Regulation of membrane-cytoskeletal interactions by tyrosine phosphorylation of erythrocyte band 3. *Blood*. 2011;117(22):5998-6006.
31. De Franceschi L, Villa-Moruzzi E, Biondani A, et al. Regulation of K-Cl cotransport by protein phosphatase 1alpha in mouse erythrocytes. *Pflugers Arch*. 2006;451(6):760-768.
32. Mallozzi C, De Franceschi L, Brugnara C, Di Stasi AM. Protein phosphatase 1alpha is tyrosine-phosphorylated and inactivated by peroxynitrite in erythrocytes through the src family kinase fgr. *Free Radic Biol Med*. 2005;38(12):1625-1636.
33. Ipsaro JJ, Huang L, Gutierrez L, MacDonald RI. Molecular epitopes of the ankyrin-spectrin interaction. *Biochemistry*. 2008;47(28):7452-7464.
34. Ipsaro JJ, Harper SL, Messick TE, Marmorstein R, Mondragon A, Speicher DW. Crystal structure and functional interpretation of the erythrocyte spectrin tetramerization domain complex. *Blood*. 2010;115(23):4843-4852.
35. Ipsaro JJ, Mondragon A. Structural basis for spectrin recognition by ankyrin. *Blood*. 2010;115(20):4093-4101.
36. Matsuoka Y, Li X, Bennett V. Adducin: structure, function and regulation. *Cell Mol Life Sci*. 2000;57(6):884-895.
37. Kolch W, Pitt A. Functional proteomics to dissect tyrosine kinase signalling pathways in cancer. *Nat Rev Cancer*. 2010;10(9):618-629.
38. Gauthier E GX, Mohandas N, An X. Phosphorylation-dependent perturbations of the 4.1R associated multiprotein complex of the erythrocyte membrane. *Biochemistry*. 2011; 50(21): 4561-4567.

39. Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. *Science*. 1991;252(5009):1162-1164.
40. Cooper RA, Durocher JR, Leslie MH. Decreased fluidity of red cell membrane lipids in abetalipoproteinemia. *J Clin Invest*. 1977;60(1):115-121.
41. Salomao M, Zhang X, Yang Y, et al. Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane. *Proc Natl Acad Sci U S A*. 2008;105(23):8026-8031.
42. Stenberg PE, Pestina TI, Barrie RJ, Jackson CW. The Src family kinases, Fgr, Fyn, Lck, and Lyn, colocalize with coated membranes in platelets. *Blood*. 1997;89(7):2384-2393.
43. Evangelista V, Pamuklar Z, Piccoli A, et al. Src family kinases mediate neutrophil adhesion to adherent platelets. *Blood*. 2007;109(6):2461-2469.
44. de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. Lipid raft-associated protein sorting in exosomes. *Blood*. 2003;102(13):4336-4344.
45. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci*. 2002;115(Pt 12):2505-2515.
46. Harder T, Scheiffele P, Verkade P, Simons K. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J Cell Biol*. 1998;141(4):929-942.
47. Salzer U, Zhu R, Luten M, et al. Vesicles generated during storage of red cells are rich in the lipid raft marker stomatin. *Transfusion*. 2008;48(3):451-462.

TABLE 1

DEMOGRAPHIC AND MOLECULAR DATA OF CONTROL AND CHOREA-ACANTHOCYTOSIS SUBJECTS

	Gender	Age (yrs)	Molecular defect/WB analysis for chorein
Controls (*n*=12)	9F/3M	35.6±2.3	-
ChAc 1	F	35	Intron 3: c.188-5T>G (splice Site); mutation on other allele unknown/ nd
ChAc 2	F	34	nd/ chorein absent
ChAc 3	F	30	Exon 37: c.4286G>C;p.A1428P; Intron 55: c.7806G>A;p.P2602P (splice site)/ chorein absent
ChAc 4	F	40	Exon 34: c.3889C>T; p.R1297X; Exon 36: c.4216del ; p.V1406CfsX20/nd
ChAc 5	M	47	Intron 22: c.2288+2T>C; (splice-site mut); Intron 61: c.8472-1G>C; (splice-site mut)/ nd
ChAc 6	F	56	(homozygous) Exon 13: c.1115del; p.K372SfsX2/nd
ChAc 7	M	32	nd/ chorein absent
ChAc 8	F	38	Intron 6 : c.495+5G>A (splice-site mut) Exon 40 : c.4903_4906del ; p.K1635VfsX6/ nd
ChAc 9	F	40	nd/ chorein absent

ChAc: chorea-acanthocytosis; WB: Western blot; F: female; M: male; yrs: years; nd: not determined

TABLE 2

PROTEINS WITH DIFFERENT DEGREES OF TYR-PHOSPHORYLATION IN RED CELL MEMBRANES FROM CONTROL AND CHOREA-ACANTHOCYTOSIS SUBJECTS

Band #	AC	Protein	Theorical protein MW (kDa)	Matching peptide	Coverage %
1	P11277	Beta spectrin_SPTB1	246.468	146	50
2	P16157	Ankyrin_ANK1	206.265	20	22
3	Q8WX82	Beta I spectrin form beta I sigma 3 (fragment)_Q8WX82	117.549	14	20
4	P02730	Band 3 anion transport protein_B3AT	101.792	16	18
5	P02730	Band 3 anion transport protein_B3AT	101.792	23	24
6	Q9H4B4	Serine threonine protein kinase PLK3_ PLK3	71.629	6	8
7	P35612	Beta adducin_ADDB	80.854	9	11
8	P11171	Band 4.1_EPB41	97.017	10	24
9	P16452	Band 4.2_EPB42	77.009	9	12
10	Q9H4B4	Serine threonine protein kinase PLK3_ PLK3	71.629	6	11
11	Q00013	55 kDa erythrocyte membrane protein_EM55	52.296	19	34
12	Q00013	55 kDa erythrocyte membrane protein_EM55	52.296	6	18
13	P47895	Aldehyde dehydrogenase family 1 member A3_AL1A3	56.108	3	6
14	P04040	Catalase_CATA	59.756	4	9
15	O94921	Serine threonine protein kinase PFTAIRE-1_CDK14	53.057	5	10
16	Q14012	Calcium calmodulin dependent protein kinase type 1 _KCC1A	41.337	4	10
17	P60709	Beta actin_ACTB	41.737	10	34
18	Q96GD4	Serine threonine protein kinase 12_AURKB	39.311	4	15
19	P04406	Glyceraldehyde-3-P-dehydrogenase_G3P	36.053	9	36
20	P27105	Stomatin_STOM	31.731	10	40
21	P00918	Carbonic Anhydrase 2_CAH2	29.246	3	11
22	Q96E17	Ras related protein Rab-3C_RAB3C	25.952	3	17

The corresponding bands are indicated in Fig. 2b. AC: accession number; PY; tyrosine (Tyr-) phosphorylation. MW: molecular weight. The success rate of protein identification by MALDI MS/MS was 82±6.7% (n=9).

TABLE 3

BIOINFORMATIC ANALYSIS OF THE DIFFERENTLY TYROSINE PHOSPHORYLATED PROTEINS IDENTIFIED IN CHOREA-ACANTHOCYTOSIS RED CELLS

#	AC	Protein	Lyn-target	Score
1	P11277	Beta spectrin_SPTB1	Y-307 Y-474 Y-493 Y-660 Y-1590	2.447 3.596 3.383 4.128 3
2	P16157	Ankyrin_ANK1	Y-884 Y-1468 Y-1750	3.83 2.362 2.447
3	Q8WX82	Beta I spectrin form beta I sigma 3 (fragment)_Q8WX82	Y-307 Y-474 Y-493 Y-660 Y-1590	2.447 3.596 3.383 4.128 3
4	P027330	Band 3 anion transport protein_B3AT	Y-8 Y-21 Y-46 Y-359 Y-904	8.809 6.553 4.149 4.702 5.915
5	P027330	Band 3 anion transport protein_B3AT	Y-8 Y-21 Y-46 Y-359 Y-904	8.809 6.553 4.149 4.702 5.915
6	Q9H4B4	Serine threonine protein kinase PLK3_ PLK3	Y-136	2.383
7	P35612	Beta adducin_ADDB	Y-377	0.66
8	P11171	Band 4.1_EPB41	Y-374	2.489
9	P16452	Band 4.2_EPB42	Y-435	2.085
10	Q9H4B4	Serine threonine protein kinase PLK3_ PLK3	Y-136	2.383
11	Q00013	55 kDa erythrocyte membrane protein_EM55	Y-331 Y-429	2.468 2.83
12	Q00013	55 kDa erythrocyte membrane protein_EM55	Y-331 Y-429	2.468 2.83
13	P47895	Aldehyde dehydrogenase family 1 member A3_AL1A3	Y-497	**1.683**
14	P04040	Catalase_CATA	Y-260	1.809
15	O94921	Serine threonine protein kinase PFTAIRE-1_CDK14	Y-135	4.17
16	Q14012	Calcium calmodulin dependent protein kinase type 1_ KCC1A	Y-20 Y-235	2.872 2.723
17	P60709	Beta actin_ACTB	Y-240	2.702
18	Q96GD4	Serine threonine protein kinase 12_AURKB	Y-8	2.319
19	P04406	Glyceraldehyde-3-P-dehydrogenase_G3P	Y-255	**1.66**
20	P27105	Stomatin_STOM	Y-60 Y-123 Y-124 Y-252	*0.213* *0.404* *0.234* *0.426*
21	P00918	Carbonic Anhydrase 2_CAH2	Y-40 Y-51 Y-114	**1.511** **1.745** **1.681**
22	Q96E17	Ras related protein Rab-3C_RAB3C	Y-17 Y-29 Y-50 Y-92 Y-99 Y-100 Y-110 Y-131	*0.809* *0.383* *0.298* *0.638* *0.596* *0.638* *0.191* *0.234*

Light grey refers to identified phosphopeptides of beta-spectrin and beta adducin (see text for details); bold refers to low threshold analysis (see Materials and Methods for details); italic refers to very low threshold analysis (see Materials and Methods for details).

FIGURE LEGENDS

Fig.1. Proteomic analysis of red cell membrane fractions shows differences in ChAc compared to healthy controls. (a) Morphology of red cells from control and chorea-acanthocytosis (ChAc) subjects. **(b-e)** Red cells from control (C) and chorea-acanthocytosis (ChAc) were fractionated in fraction 1 (F1) corresponding to a density < 1.074, containing reticulocytes, and fraction 2 (F2) corresponding to a density > 1.092, containing acanthocytes. **(b)** The fractionated red cell membrane proteins were separated by one-dimensional electrophoresis (1DE) and stained with colloidal Coomassie blue. The bands that were identified by mass-spectrometry are indicated: β spectrin (accession number: P11277) 48 % coverage; α spectrin (accession number: P02549) 52 % coverage; band 3 (accession number: P027330) 25% coverage; band 4.1R (accession number: P11171) 22 % coverage; band 4.2 (accession: P16452 number) 18 % coverage; β-actin (accession number: P60709) 26 % coverage; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; accession number: P04406) 18 % coverage; peroxiredoxin 2 (Prx2; accession number: P32119) 32% coverage. The figure shows a representative of nine experiments performed with similar results. **(c)** Western-blot (Wb) analysis of red cell membranes separed by 1DE with specific antibodies against peroxiredoxin 2 (Prx2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), flotillin-1 and stomatin proteins of fractionated red cells from controls (C) and chorea-acanthocytosis (ChAc) subjects. Actin was used as loading control. The data are representative for eight experiments. **(d-e)** The membrane proteins of fractionated red cells (see above and Method section) were separated by two-dimensional electrophoresis (2-DE). Twin 2DE gels were run: one stained with colloidal Coomassie and the other transferred to membrane for Western-blot analysis. **(d)** The colloidal Coomassie blue stained gels underwent image analysis and differently expressed proteins were identified by mass-spectrometry (see Method and Methods and Supplementary Methods). Red and green spots indicated the differently expressed proteins based on image analysis. The identified proteins are reported in Table 2S. The figures show one representative experiment from a total eight experiments performed. **(e)** Western-blot (Wb) analysis of the 2DE maps with specific anti- protein p55 antibody on F1 red cells from control and ChAc subjects (validating the differently expressed spots 32C, 33C, 34Ci n Fig. 1d, Table 2S), anti-flotillin-1 antibody on F1 red cells from control and ChAc subjects (validating the differently expressed spots 40C in Fig. 1d, Table 2S), anti-protein 4.1R antibody on F2 red cells from control and ChAc subjects

(validating the differently expressed spots 64C-68C in Fig. 1d, Table 2S). The figures show one representative experiment of three experiments performed with similar results.

Fig. 2. Tyrosine phosphorylation of red cell membrane protein is increased in chorea-acanthocytosis compared to normal controls. Red cells from control (C) and chorea-acanthocytosis (ChAc) subjects were fractionated as described in Materials and Methods and in the legend of Fig. 1b-e. Western-blot (Wb) analysis with specific anti-phosphotyrosine (PY) antibodies of red cell membrane proteins separated by either bidimensional electrophoresis (2DE) or monodimensional electrophoresis (1DE). **(a)** Twin 2DE gels were run: one used for colloidal Coomassie stained gels (see Fig. 1d) and the other for the Western-blot analysis with specific anti-phosphotyrosine (PY) antibodies. **Upper panel:** membranes of high density red cell fraction (F2) from control (C) and chorea-acanthocytosis (ChAc) subjects are shown. Similar results were also obtained in low density red cell fraction (F1) from control and ChAc (data not shown). **Lower panel:** Dephosphorylation of blotted proteins by recombinant λ protein phosphatase (400 U/mL). The blotted membranes were incubated in TBS containing 1% BSA, 0.1 % TRITON X-100, 2 mM $MnCl_2$ (overnight at 4°C). and then probed with anti-phosphotyrosine antibodies. The data are representative for three experiments with similar results. **(b)** 1DE gels (13 cm) were blotted for Western-blot (Wb) analysis with specific anti-phosphotyrosine (PY) antibodies. The bands with different staining intensities were identified by mass spectrometry (Table 2) (see also Materials and Methods section and Supplementary Methods). Actin was used as a loading control. The data are representative for eight experiments, see also Fig. 1Sb for densitometric analysis of the Tyr-phosphorylation profile of the red cell membrane proteins.

Fig. 3. ChAc red cells show increased Lyn tyrosine kinase associated to the membrane. Red cells from control (C) and chorea-acanthocytosis (ChAc) were fractionated as described in Materials and Methods and in the legend of Fig. 1b-e. **(a)** Western-blot (Wb) analysis with specific antibodies of membrane associated tyrosine kinase Lyn and phospho-Lyn (p-Lyn), Syk and phospho-Syk (p-Syk). Actin was used as a loading control. Shown is a representative of six experiments. Vertical line(s) have been inserted to indicate a repositioned gel lane. **(b)** Western-blot analysis with specific antibodies against Tyr-8 on the N-terminal of band 3, as a Syk target, and Tyr-904 on the transmembrane domain of band 3 (as a Lyn target). We used normal red cells treated with Na-vanadate (see Materials and Methods) as positive controls. Total band 3 was used as loading control. **(c)** Western-blot (Wb) analysis with specific anti-phosphotyrosine (PY)

antibodies of high density fraction (F2) of red cells from control (C) and chorea-acanthocytosis (ChAc) subjects are shown. The F2 red cells wre incubated with or without the Src family kinase inhibitors PP1 (10 μM) and PP2 (10 μM) as previously reported.[19] The arrows indicated the bands affected by PP1-PP2 treatment in ChAc red cells compared to untreated ChAc red cells. The data are representative for three experiments (on 6 cm gels). Actin was used as a loading control. The data are representative for three experiments. Similar results were also obtained with cells from low density fraction (F1) control and ChAc red cells (data not shown).

Fig. 4. Modes of interaction of Lyn with membranes from ChAc red cells. (a) Membranes of F2 red cells from control (C) and chorea-acanthocytosis (ChAc) subjects were extracted with Triton X-100 or NaCl (see Materials and Methods) and assayed after ultracentrifugation for the presence of Lyn by Western-blot (Wb) analysis. The data shown here are obtained with F2 ChAc red cells. Similar data were obtained with F1 control and ChAc red cells (data not shown). (**b)** Membranes of F2 red cells from control (C) and chorea-acanthocytosis (ChAc) subjects were incubated in the presence or absence of GST-Lyn/SH3, GST-Lyn/SH2, cdb3 or phospho (p-)-cdb3 and assayed after ultracentrifugation for Lyn in the resulting soluble (S) and pellet (P) fractions for the presence of Lyn by western-blot (Wb) analysis. Similar data were obtained from F1 control and ChAc red cells (data not shown). The membranes were reprobed with anti-actin antibody as loading control.

Fig.5. **Phosphorylation patterns of normal and ChAc red cells membranes by exogenous Lyn.** Membranes of high density fraction (F2) of red cells from control (C) and chorea-acanthocytosis (ChAc) subjects were incubated without (lanes 1 and 5), or with exogenous Lyn (lanes 2-4 and 6-8) and with GST-Lyn/SH3 (lanes 3 and 7) or GST-Lyn/SH2 (lanes 4 and 8), respectively. Samples were then analyzed by Western-blot (Wb) analysis with anti-phosphotyrosine antibodies. Similar data were obtained with F1 control and ChAc red cells (data not shown). The membranes were reprobed with anti-actin antibody as loading control.

Fig.6. Effect of phosphorylation of red cells membranes by Syk and/or Lyn on band 3 binding to β-adducin in normal and ChAc red cell membranes. (a) Membranes (lanes 1 and 2), or Syk-phospho-membranes (lanes 3 and 4) of F2 red cells from control (C) were incubated without (lanes 1 and 3), or with exogenous Lyn (lanes 2 and 4) and subjected to Western blot analysis with anti-phospho-Tyr (anti-pTyr) antibody (upper panel) or were extracted with Triton X-100 and assayed after ultracentrifugation for the

presence of band 3 by Western-blot analysis (lower panel). The membranes were reprobed with anti-actin antibody as loading control. **(b)** Band 3 was immunoprecipitated from membranes (lanes 1 and 2), and Syk-phospho-membranes (lanes 3 and 4) of F2 red cells from C after incubation without (lanes 1 and 3), or with exogenous Lyn (lanes 2 and 4). The immunoprecipitates were subjected to Western blot analysis with anti-pTyr (top panel), anti-β-adducin antibody. The blots were also probed with anti-band 3 antibody. **(c)** Band 3 was immunoprecipitated from membranes of F2 ChAc red cells previously incubated without or with the inhibitor PP2 (10 μM). The immunoprecipitates were subjected to Western blot analysis with, anti-β-adducin antibody. The blots were also probed with anti-band 3 antibody. The figure is representative of three independent experiments.

Fig. 1

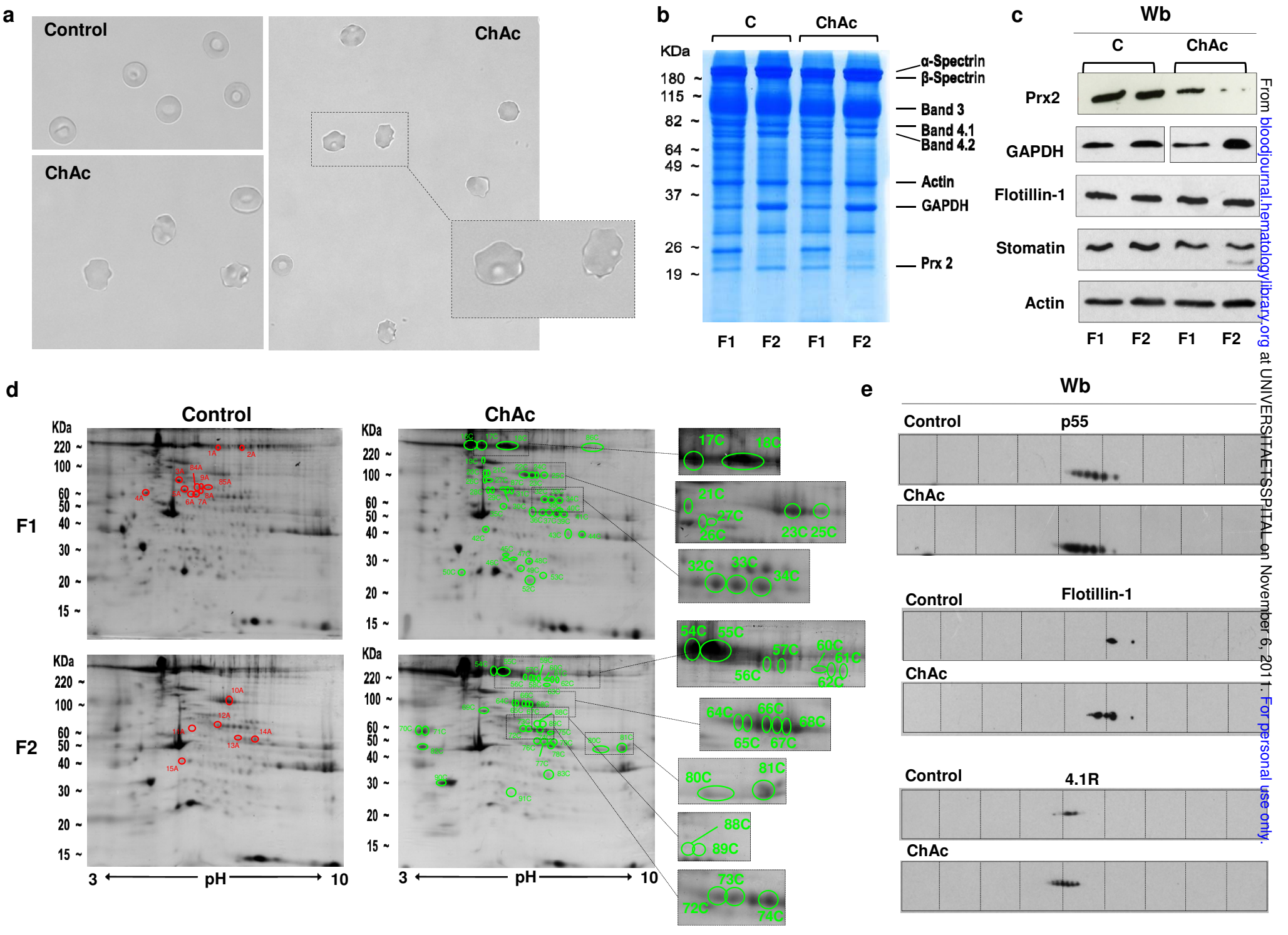
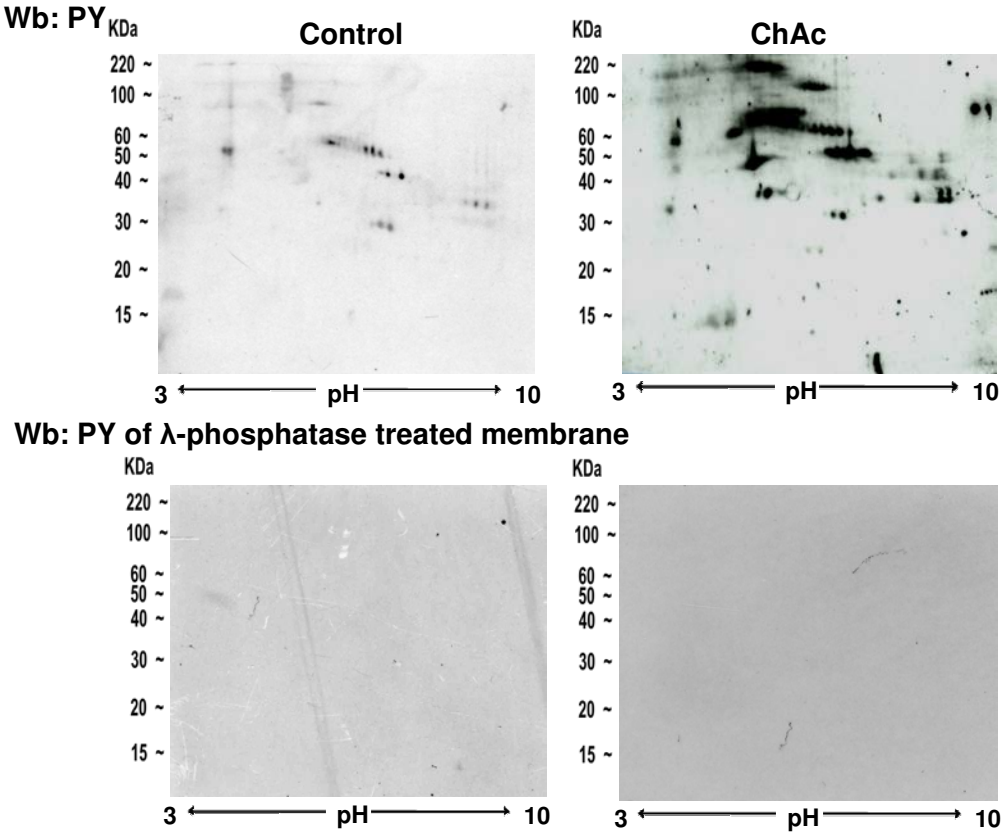


Fig. 2

a


Wb: PY
KDa
Control
ChAc
220
100
60
50
40
30
20
15
3
pH
10
Wb: PY of λ-phosphatase treated membrane

b

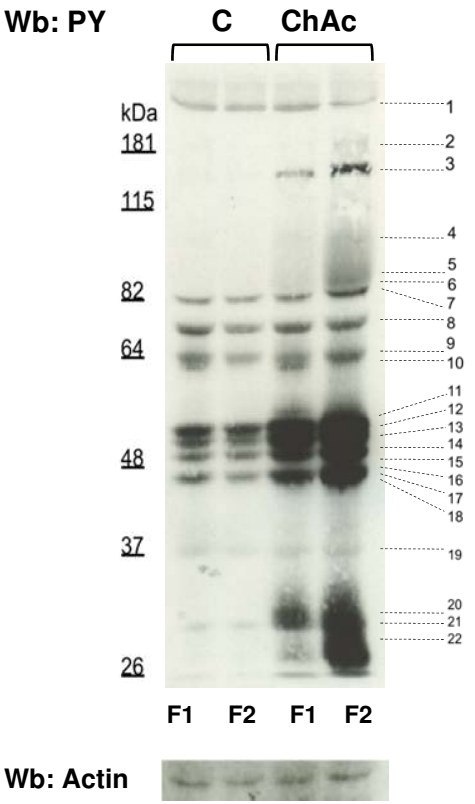

Wb: PY
C
ChAc
kDa
181
115
82
64
48
37
26
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
F1
F2
F1
F2
Wb: Actin

Fig. 3

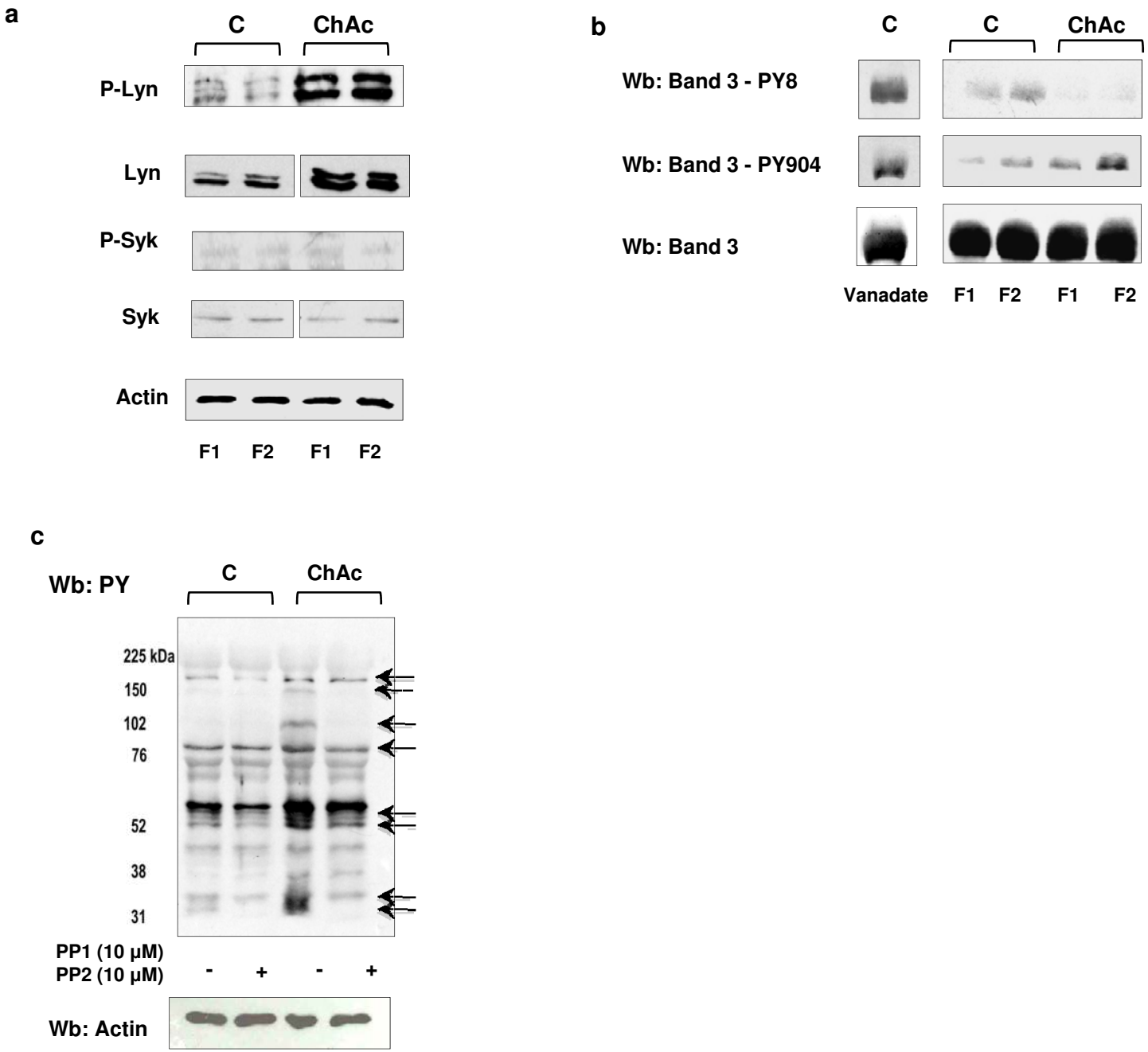

a
C
ChAc
P-Lyn
Lyn
P-Syk
Syk
Actin
F1
F2
F1
F2
b
C
C
ChAc
Wb: Band 3 - PY8
Wb: Band 3 - PY904
Wb: Band 3
Vanadate
F1
F2
F1
F2
c
Wb: PY
C
ChAc
225 kDa
150
102
76
52
38
31
PP1 (10 µM)
PP2 (10 µM)
-
+
-
+
Wb: Actin

Fig.4

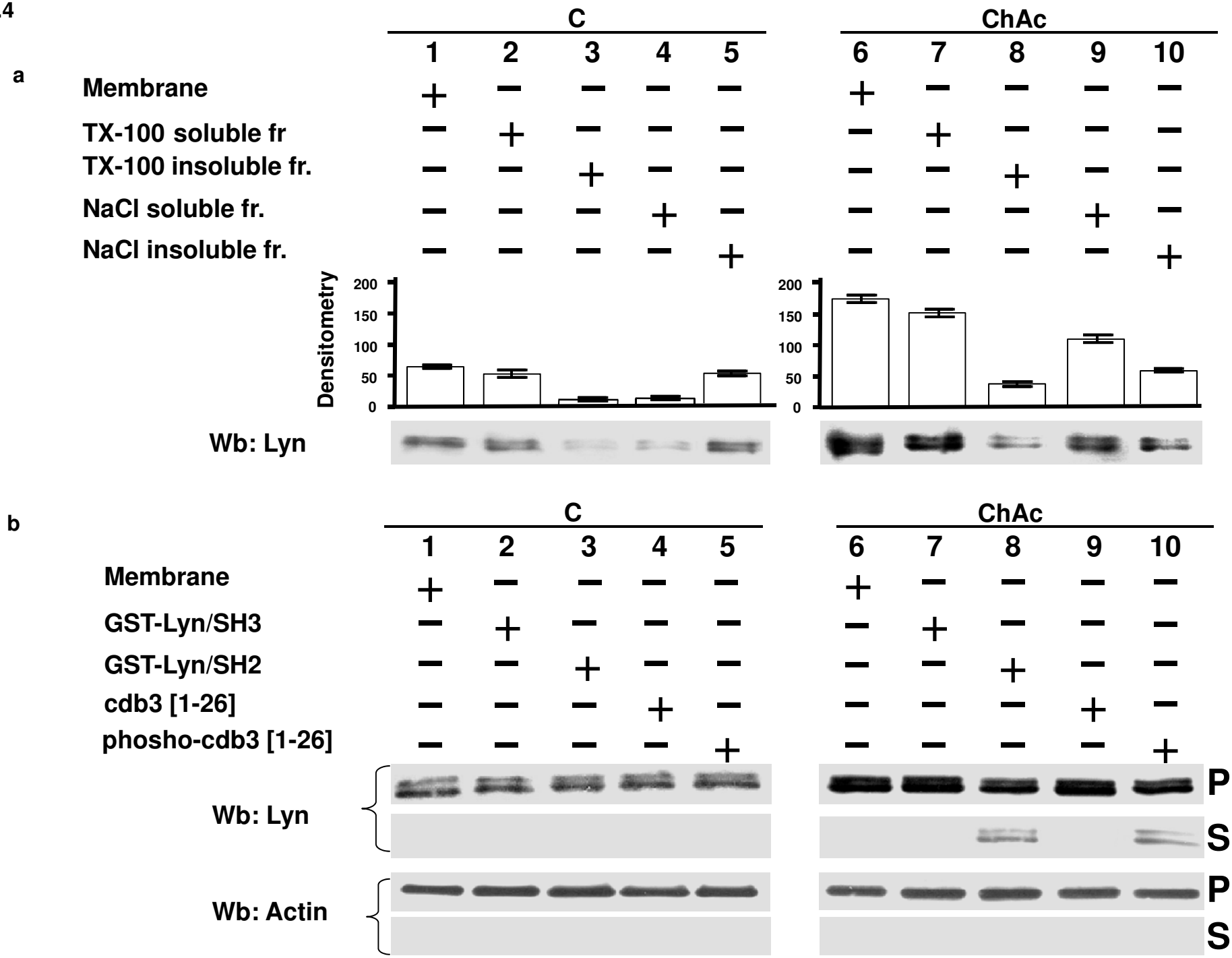

a
C
ChAc
1 2 3 4 5
6 7 8 9 10
Membrane
TX-100 soluble fr
TX-100 insoluble fr.
NaCl soluble fr.
NaCl insoluble fr.
Densitometry
200
150
100
50
0
Wb: Lyn
b
C
ChAc
1 2 3 4 5
6 7 8 9 10
Membrane
GST-Lyn/SH3
GST-Lyn/SH2
cdb3 [1-26]
phosho-cdb3 [1-26]
Wb: Lyn
Wb: Actin
P
S
P
S

Fig.5

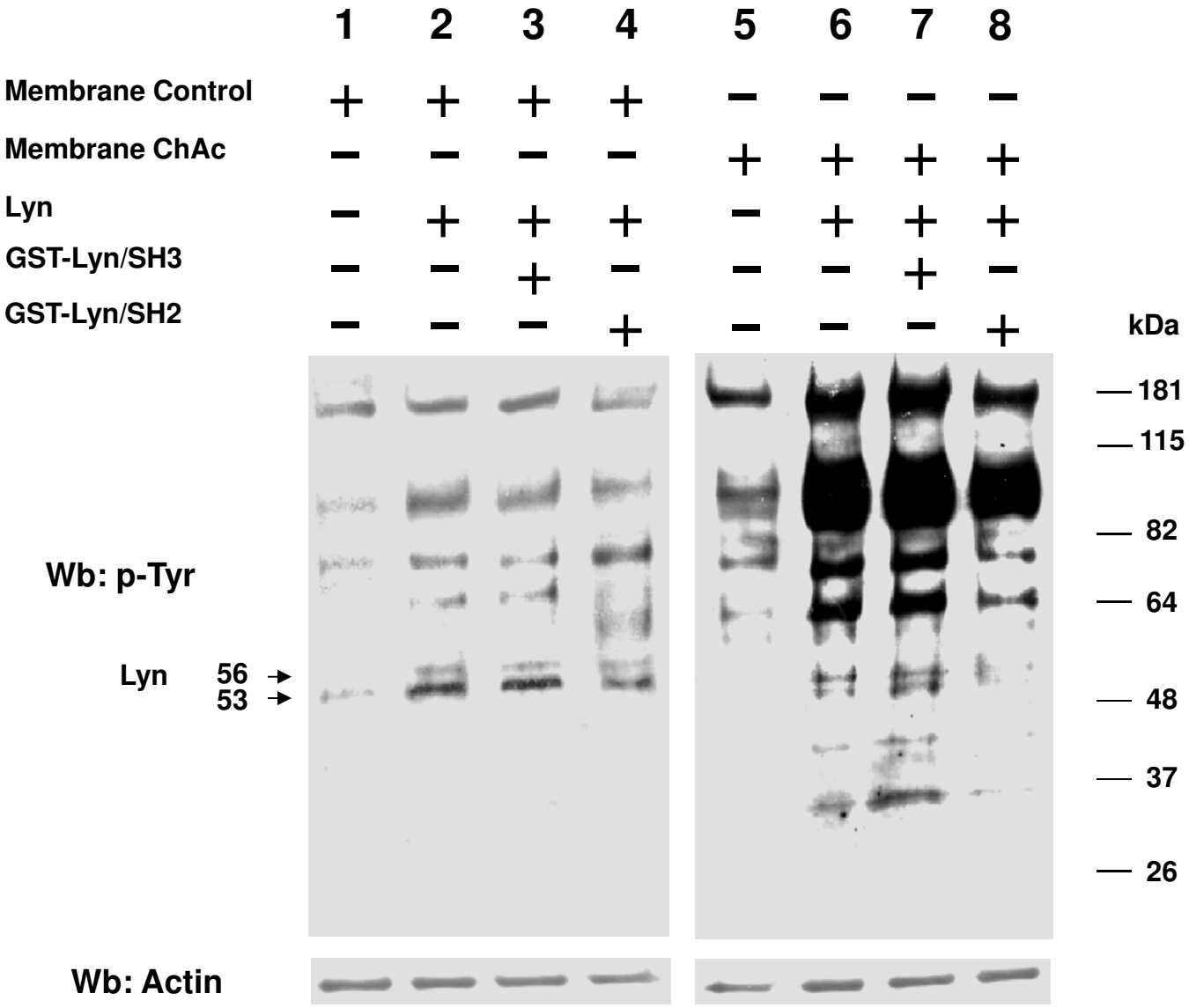
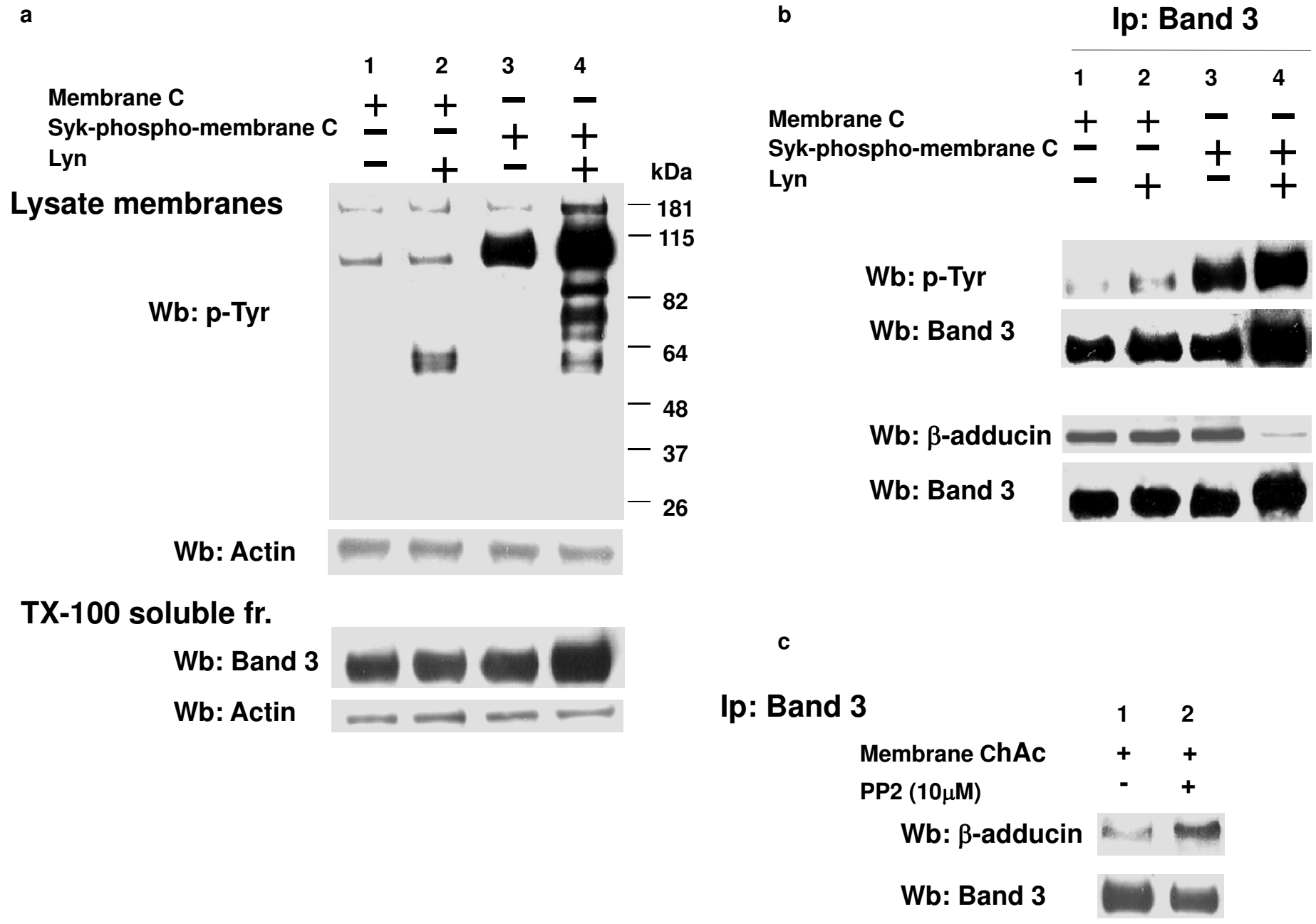

1 2 3 4 5 6 7 8
Membrane Control + + + + − − − −
Membrane ChAc − − − − + + + +
Lyn − + + + − + + +
GST-Lyn/SH3 − − + − − − + −
GST-Lyn/SH2 − − − + − − − +
kDa
181
115
82
64
48
37
26
Wb: p-Tyr
Lyn 56 53
Wb: Actin

Fig.6


a
1
2
3
4
Membrane C
Syk-phospho-membrane C
Lyn
kDa
Lysate membranes
181
115
Wb: p-Tyr
82
64
48
37
26
Wb: Actin
TX-100 soluble fr.
Wb: Band 3
Wb: Actin
b
Ip: Band 3
1
2
3
4
Membrane C
Syk-phospho-membrane C
Lyn
Wb: p-Tyr
Wb: Band 3
Wb: β-adducin
Wb: Band 3
c
Ip: Band 3
1
2
Membrane ChAc
PP2 (10μM)
-
+
Wb: β-adducin
Wb: Band 3